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ELECTROPHORETIC CHARACTERISTICS OF OUTER MEMBRANE PROTEINS OF *NEISSERIA MENINGITIDIS* (U)

by

A. Rashid Bhatti
and
Elena Jascolt

Project No. 351SD

July 1987

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ABSTRACT

Eight strains of *Neisseria meningitidis* belonging to different serogroups were analyzed for their virulence in mice and their release of outer membrane proteins (OMP) into the growth medium during growth. All strains released proteins. No detectable lipopolysaccharide was observed, however, SDS-PAGE showed a heterogenicity in the protein number and profile among the different strains of *N. meningitidis* tested. No Class I protein was released by any strain investigated, suggesting that Class I proteins may be tightly bound to the outer cell wall complex.

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INTRODUCTION

The genus Neisseriae describes a group of non-motile, non-spore forming Gram-negative cocci, which are chemo-organotropic aerobic or facultative microbes, producing catalase and cytochrome oxidase (Morello and Bohnoff, 1980; Reyn, 1974). These bacteria have complex growth requirements (Morello and Bohnoff, 1980; Reyn, 1974), and demonstrate species diversity with respect to nutritional requirements (Catlin, 1973).

Six species are described by Reyn (1974), and two of these, *Neisseria gonorrhoeae* and *Neisseria meningitidis*, are pathogenic for humans. Both of these species continue to be important and common aetiological agents of sexual and nasopharyngeal infections in humans. Pathogenic strains of *N. meningitidis* are always encapsulated while carrier strains are often nonencapsulated (Peltola, 1983; Frasch, 1979). The immunochemical nature of the capsular polysaccharide determines the meningococcal serogroup. Five of the 11 serogroups are associated with over 98% of meningococcal disease (Peltola, 1983).

The bacteria excrete growth inhibitory substances (Morse, 1979; Walstad *et al.*, 1974) and are sensitive to autolysis (Morse, 1979). The organisms have an optimal growth temperature between 35° and 37°C and often grow best in a CO_2 enriched atmosphere (Cruickshank *et al.*, 1973; Morello and Bohnoff, 1980; Reyn, 1974).

Although much has been written about this pathogen and the variety of clinical manifestations of meningococcal disease, little is known about the direct relationship between the physiology and biochemistry of the microbe, and the pathogenesis of the disease. A number of virulence factors, including an antiphagocytic capsule, pili, different proteases and lipopolysaccharides (LPS) have been demonstrated in *N. meningitidis* (DeVoe, 1982; O'Reilly and Bhatti, 1986; Hassan, Bhatti and White, 1984; Morse, 1979; Bhatti *et al.*, 1982; Jennings *et al.*, 1975; Andersen and Solberg, 1978).

One particular area of interest has been the association between the virulence of an organism and its proteins. Since the outer membrane is usually available for direct contact between a pathogen and its host, one might suspect it to play a role in pathogenic processes (Darveau, Charnetzky and Hurebert, 1980). Frasch, McNelis and Gotschlick (1976), have shown that outer membranes prepared from various serotypes have distinct protein profiles after sodium dodecyl sulfate polyacryamide gel electrophoresis (SDS-PAGE). Each of the serotypes appeared to possess a different set of three to four major outer membrane proteins (OMP) (Frasch, Tsai and Mocca, 1986).

This present study was undertaken to compare and characterize the proteins and glycoproteins which are released into the medium during the growth of various strains of *N. meningitidis*. Since these strains differ in their degree of virulence, the electrophoretic characterization of these molecules may provide an insight into the role they play in pathogenicity.

MATERIALS AND METHODS

Bacterial Strains

N. meningitidis strains, DRES-03, -04, -22, -32, -33, -30, and -14, used in this study were isolated at the Defence Research Establishment Suffield (DRES). Stock cultures were prepared by lyophilizing strains after 1 to 3 initial subcultures and storing these at 4°C. Strain M-1011 was obtained from Dr. C.E. Frasch, Center for Drugs and Biologics, FDA, Bethesda, Maryland.

Infectious Study

Infectious studies using the different strains of *N. meningitidis* was carried out as described previously (Bhatti, *et al.*, 1982).

Preparation of Cell Free Supernatants (CFS)

One lyophilized vial of each strain was rehydrated in 9.0 mL of Neisseria Defined Medium (NDM) (Archibald and DeVoe, 1978). This was used to seed a tissue culture flask containing NDM agar. The flasks were incubated at 35 – 37°C for 18 h in a 5% CO_2 atmosphere (v/v).

The cells were removed from the agar surface using sterile glass beads and 9.0 mL of NDM. Bacterial suspension (2.0 mL of each) were inoculated into 100 mL of NDM in 500 mL Erlenmyer flasks. The flasks were incubated at 35°C in 5% CO_2 atmosphere with constant rotatory shaking in an Incubator Shaker — Model G26 (New Brunswick Scientific Co., Inc., New Brunswick, N.J.) at 125 rpm for 7 h.

After 7 h incubation, 0.1 mL of this growth was transferred to another flask containing 100 mL of NDM and incubated for 18 h under the same conditions. The culture was centrifuged at 15,000 rpm in a J-21 Beckman centrifuge using a JA-20 rotor for 10 min, after which the Cell Free Supernatant (CFS) was removed, filter sterilized and refrigerated at 4°C. Ninety mL of this CFS was lyophilized.

To remove any residual salts from the CFS, the lyophilized material was resuspended in a minimum volume of distilled water (approximately 5 mL) and dialyzed for 36 h against distilled water.

This material was centrifuged at 10,000 rpm in a JA-21 head for 15 min using the Beckman JC-21 centrifuge and the supernatant decanted and retained. The pellet was washed once with water and centrifuged again. Finally, this pellet (insoluble fraction) was suspended in 1 mL of 0.1 N NaOH and refrigerated at 4°C.

The soluble fraction (supernatant) was frozen in dry ice-ethanol and lyophilized overnight. The dried material was resuspended in 1.0 mL of distilled water and frozen for further use.

Analytical Techniques

Protein Estimation: The method of Lowry *et al.*, (1951) was used to estimate protein concentrations. Bovine serum albumin was employed as a standard.

Enzyme Assay: Glucose-6-phosphate dehydrogenase activity was assayed as described (Bhatti, DeVoe and Ingram, 1976).

Lipopolysaccharide Determination: Total LPS, as 2-keto-3-deoxyoctonate (KDO), was estimated colorimetrically by the thiobarbituate method of Osborn (1963) using purified *Escherichia coli* LPS as a standard.

Electrophoresis

Sodium dodecyl sulfate — polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 11% gels using the discontinuous tris (hydroxymethyl) aminomethane-HCl buffer system of Laemmli (1970) in a vertical slab gel apparatus (Biorad Protein Dual Slab Cell, Biorad Laboratories, Mississauga, Ontario).

To obtain optimal separations and band resolution, the porosity, pH, and TRIS and glycine concentrations in the stacking and separating gels were investigated. The

following procedures and conditions were found to yield optimal results. Gels containing 6% (stacking gel), and 11% (resolving gel) acrylamide were prepared from a monomer stock of 30% (w/v) acrylamide (Sigma Chemical Company, St. Louis, Missouri) and 0.8% (w/v) N,N-methylene-bis-acrylamide (Sigma Chemical Co.). The gels were polymerized chemically by the addition of 0.025% (v/v) tetramethylethylenediamine (TEMED) (Biorad Laboratories) and ammonium persulfate (Biorad Laboratories). Gels of 1.5 mm thickness, 12.5 cm width and 9.5 cm length were prepared using glass plates as a mold. The stacking gels were 1.7 cm in length and contained, 0.375 M Tris-HCl (pH 8.8) and 0.1% SDS. The electrode buffer (pH 8.3) consisted of 0.025 M Tris-HCl, 0.1824 M glycine (Fisher Scientific Co., Fairlawn, New Jersey) and 0.1% SDS (Biorad Laboratories).

Samples were mixed with an SDS-PAGE derivitization buffer containing 0.05 M Tris-HCl (pH 6.8), 10% SDS, 10% glycerol, a pinch of bromophenol blue, and 1% 2-mercaptoethanol, such that all aliquots contained approximately 20 μg protein. Samples were heated at 100° C for 5 min and 50 μL aliquots applied to the slab gel. High and low molecular weight standards (Biorad Laboratories) were prepared similarly. Electrophoresis was carried out at 4° C and 60 volts (20 mA) until the sample had run into the separating gel (about 1½ h) and then 120 volts (30 mA) until the dye front migrated to the bottom of the gel (about 3 h).

Staining Of Gels

Coomassie Brilliant Blue/Silver Stain: Upon completion of electrophoresis, gels were stained for 1 h in a solution of Coomassie brilliant blue R250 (Sigma Chemical Co.) (0.125% Coomassie brilliant blue, 9.2% acetic acid and 50% methanol). Gels were destained in several changes of 40% methanol/10% acetic acid at 37° C, until the background was clear, and then incubated in two changes of 10% ethanol/5% acetic acid (v/v) for a total of 1 h at room temperature. The gels were then stained with silver (silver staining kit, Biorad Laboratories) according to the instructions given by the supplier. This silver stain is derived from the method of Merril, Goldman and Keusen (1982). Briefly, the gels were rinsed with triple distilled water and immersed for 10 min in the oxidizing solution, followed by extensive washing in triple distilled water until all excess oxidizer was

removed (indicated by a colorless background). The gels were then immersed for 30 min at room temperature in silver reagent followed by a 10 min wash in triple distilled water. Cels were then incubated in two to three changes of developer until the desired band intensity was reached. Development was stopped with 5% acetic acid.

Periodic Acid-Schiff (PAS): Gels were fixed and stained in the dark with PAS reagent according to Segrest and Jackson (1972). Upon completion of electrophoresis, the gels were incubated overnight in fixative (40% ethanol/5% acetic acid), treated with 1% periodic acid (in 7% acetic acid) for 2 h, then decolorized in 1% sodium bisulfite (in 0.1 N HCl) for 1 h. Several changes of sodium bisulfite were required to decolorize the gels completely. The gels were then rinsed with distilled water and incubated in Schiff's reagent (Sigma Chemical Company) for 24 h at room temperature. Pink coloration indicated positively stained glycoproteins. To intensify the color of the stain, the gels were incubated further in 0.2% potassium metabisulfite in 5% acetic acid for 2 h and then stored in 6% acetic acid at 4°C.

RESULTS AND DISCUSSION

The proteins present in the outer membrane of meningococci are of considerable interest, being implicated in the host bacteria interactions which determine the course of an infection. Knowledge of the structural relationships between the OMP should lead to a further understanding and explanation, in molecular terms, of the factors which contribute to the variations in pathogenicity (Heckels, 1981; Holbein, 1980). Frasch *et al.*, 1986, have reported considerable variation between strains of different serotypes in their OMP profiles on SDS-PAGE.

SDS-PAGE has been a useful tool to study proteins produced by many Gram-negative bacteria (Mintz, Epicella and Morse, 1984; Frasch *et al.*, 1986). In the present study, SDS-PAGE followed by both Coomassie blue staining and silver staining, was used to study some of the physical and structural properties of meningococcal proteins. The results of this study are shown in Figures 1 and 2. There appeared to be considerable variations between strains in their OMP as shown by SDS-PAGE as well as between the

soluble and insoluble fractions of the outer membrane of the same strain of *N. meningitidis*. Generally, more polypeptide bands were found in the soluble fraction as compared to the insoluble fractions.

Major OMP have been divided into five different structural classes, corresponding roughly to their apparent molecular weights on SDS-PAGE (Frasch *et al.*, 1986). The class 5 proteins are the so-called heat modifiable proteins similar to those found in gonococci and *E. coli* (Frasch and Mocca, 1978; Hindennach and Henning, 1975; Heckels, 1977; Stephens and McGee, 1983), while the class 2 and 3 proteins are the meningococcal equivalent to the porins found in other Gram-negative bacteria (Lugtenberg and Van Alphen, 1983). Stephens and McGee (1983) have associated the virulence of *N. meningitidis* with a low molecular weight (MW) OMP.

OMP released by the tested strains were classified according to their apparent MW's and are presented in Table 1. None of the strains released class 1 protein. DRES-03, -04, -22, and -33 released proteins of classes 2, 3 and 5. DRES-32 released proteins of classes 2, 4 and 5. DRES-30 and M-1011 released class 3 and 5 proteins, while DRES-14 released only class 3 protein.

Soluble fraction samples contained proteins with molecular weights of 41,000 and 28 – 31,000 (Fig. 1). Except for strain DRES-14, a number of proteins of less than 32,000 MW were found in each strain. Exceptions to this were the insoluble fractions of *N. meningitidis* strain DRES-33, and M-1011, for which no protein bands were detected.

Our results suggest that class 1 proteins are most probably tightly bound to the cell surface and are not released into the medium during growth. The heterogenicity in the OMP released into the medium during growth coupled with the wide range of virulence exhibited by the various *N. meningitidis* strains (Table 2) would suggest that not all of these proteins are involved in pathogenic processes. These findings are not in complete agreement with those previously reported for outer membrane vesicles of *N. meningitidis* (Frasch and Peppler, 1982; Frasch *et al.*, 1986; Stephens and McGee, 1983).

As the degree of pathogenicity increased (as determined by LD_{50}), fewer proteins were observed in the samples. In the avirulent strain, DRES-03, there were 20 protein bands present, whereas, the highly virulent strains, DRES-14, M-1011, DRES-30,

DRES-33, and DRES-22 contained 1, 2, 2, 3 and 4 distinct proteins respectively.

Meningococci also demonstrate a high degree of release of outer membrane vesicles (blebs) which may also contain proteins. These outer membrane blebs appear to be an important factor in the pathogenicity of meningococcal disease since they contain endotoxin and are involved in the antibody induction process (Frasch and Peppler, 1982; DeVoe and Gilchrist, 1973). In the present study, we did not observe any blebs in the CFS. However, the possiblity of soluble and non-sedimentable LPS was not ruled out.

Identification of lipopolysaccharides (LPS) was attempted using the silver stain (Fig. 2). A number of bands were visualized. However, when compared to previously stained gels for protein (Coomassie blue) (Fig. 1), we found them to be almost identical. Only a few additional bands were visualized. In spite of the fact that polysaccharides have been visualized in outer membrane extracts of *N. meningitidis* (Russel and Johson, 1975), using the periodate Schiff reagent staining procedure (Segrest and Jackson, 1972), we were barely able to visualize any glycoproteins or LPS. Other analytical methods also failed to indicate detectable LPS. Therefore, silver nitrate staining coupled with analytical estimation of LPS suggest that our OMP preparations were free of LPS. The additional bands which appeared after silver nitrate staining of the same gel (Fig. 2) could be due to the weaker protein bands becoming visible due to the sensitive silver nitrate staining.

Protein secretion occurs more commonly in Gram-positive than Gram-negative bacteria. This property may be a reflection of the complex outer membrane structures of Gram-negative organisms (Costerton *et al.*, 1974). The secretion of extra-cellular protein(s) by bacteria occurs in the absence of cell lysis and probably involves a highly selective permeation process (Bhatti and Ingram, 1982). In the present study, it was demonstrated that different proteins were released into the culture medium by *N. meningitidis* strain during growth and that cell lysis does not account for their release, since the cytoplasm-located enzyme, glucose-6-phosphate dehydrogenase, was not detected in the CFS (Bhatti *et al.*, 1976).

The present study has demonstrated that both virulent and avirulent strains of *N. meningitidis* release OMP into the growth medium in the absence of cell lysis. However, the number of proteins released into the growth medium, during growth, varies among

the different strains. One strain, DRES-14, released only class 3 protein which is present in all the strains. Purifications and characterization of this apparently common class of protein, at the molecular and immunological level, may further elucidate the nature of these proteins in the virulence of this organism and its interaction with host defence mechanisms.

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Table 1

CLASSIFICATION OF *N. MENINGITIDIS* OUTER MEMBRANE PROTEINS BASED ON MOLECULAR WEIGHT

STRAINS	NUMBER POLYPEPTIDES	CLASS 1 45 – 47 × 10^3	CLASS 2 40 – 42 × 10^3	CLASS 3 37 – 39 × 10^3	CLASS 4 32 – 34 × 10^3	CLASS 5 26 – 29 × 10^3
DRES 22	4		41.0	35.0		28.7 28.0
DRES 30	2			36.0		28.0
DRES 03	7		42.5 40.0	35.0 36.0 38.0		21.0 29.0
M-1011	2			39.0		29.0
DRES 04	4		41.0	35.0		27.5 29.9
DRES 14	1			38.0		
DRES 32	3		40.0		31.0	28.0
DRES 33	3		40.5	38.2		28.0

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Table 2

VIRULENCE OF DIFFERENT STRAINS OF *N. MENINGITIDIS* IN MICE

STRAIN	SEROGROUP	LD_{50} (CFU)	VIRULENCE	PROTEINS (CLASS)
DRES 22	A	8.7×10^4	HIGH	2, 3, 5
DRES 30	A	5.5×10^3	HIGH	3, 5
DRES 03	B	$> 10^8$	NONE	2, 3, 5
M-1011	B	2.7×10^3	HIGH	3, 5
DRES 04	C	$> 10^8$	NONE	2, 3, 5
DRES 14	C	3.6×10^1	HIGH	3
DRES 32	W	1.1×10^7	LOW	3, 4, 5
DRES 33	W	1.6×10^3	HIGH	2, 3, 5

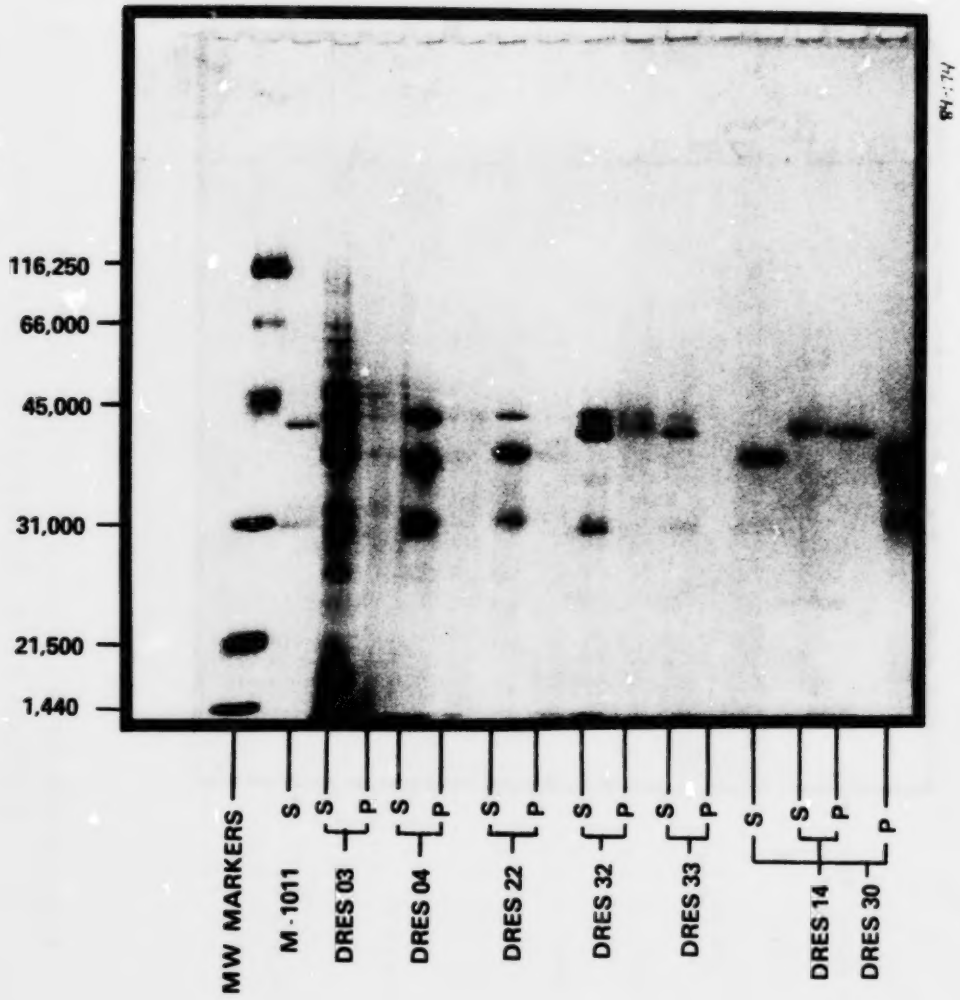

Figure 1

ELECTROPHORETIC PROFILE OF OUTER MEMBRANE PROTEINS RELEASED INTO THE GROWTH MEDIUM BY *N. MENINGITIDIS*: COOMASSIE BLUE STAIN

S, Soluble Proteins; P, Insoluble Proteins

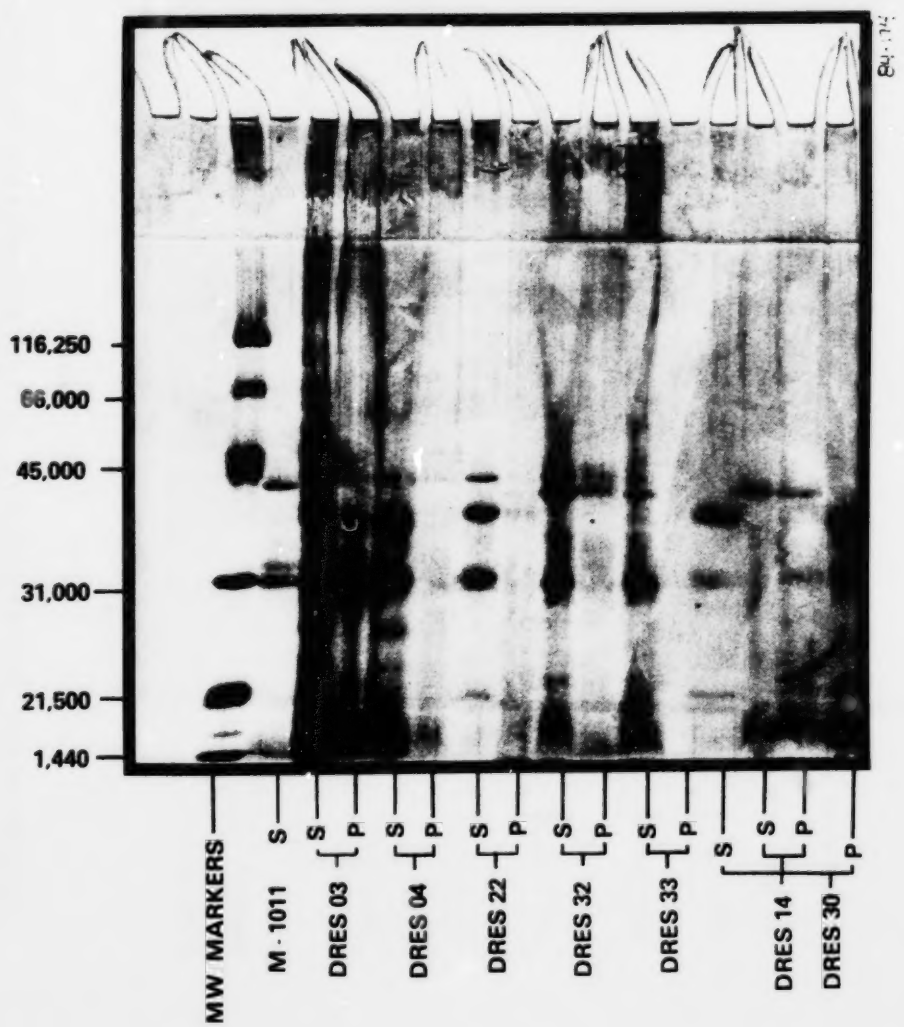

Figure 2

ELECTROPHORETIC PROFILE OF PROTEIN RELEASED INTO THE GROWTH MEDIUM BY *N. MENINGITIDIS*: SILVER NITRATE STAIN

S, Soluble Proteins; P, Insoluble Proteins